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ACID PHOSPHATASE DURING THE LIFE CYCLE OF THE NEMATODE, *PANAGRELLUS SILUSIAE*

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Since the late 1920's many theories have been suggested as possible explanations for aging, but little agreement seems to exist regarding its true nature.

Strehler (1962) proposed that aging must be universal, occurring in all old animals of a species, and essentially absent in the very young; time dependent, progressing gradually in an individual and in the population; intrinsic, due to the action of time on the biological system, rather than the result of a pathology or accident; and deleterious, unfavorably affecting the survival capacity of the individual organism in its normal environment.

Based on the concept that aging is a universal phenomenon among metazoans, Gershon (1970) considered the nematode to be suitable for aging studies, because: first, it is possible to obtain age-synchronized populations of nematodes and establish survival curves; secondly, the nematode's life-span and growth are not altered by up to 90% inhibition of DNA synthesis; and thirdly, they yield large populations under easily controlled environmental conditions, thus making them fit for biochemical investigations.

Since nematodes are eutelic organisms, cell division and turnover are negligible and most cells are already differentiated after hatching (Hyman, 1951), making any deteriorative processes leading to senescence more easily observable. The nematode chosen for this study was the free-living form, *Panagrellus silusiae*.

The lysosome has been implicated as part of the terminal lytic aging process (Brock and Strehler, 1968; Herold and Meadow, 1970; Hochschild, 1971). The purpose of this study was to investigate the lysosomal enzyme, acid phosphatase, and its isozyme patterns in the life of *P. silusiae*. The next step will be to relate these isozyme changes to a later study centering on aging.

Materials and Methods

The stock cultures of *Panagrellus silusiae* were maintained at 23–25° C. The growth medium was Gerber Mixed Cereal, which was mixed with distilled water in a weight to volume ratio of 1 : 5. Each culture was maintained for 14 days. A dilute antibiotic solution of 0.6 μg penicillin-G and 10 μg streptomycin/ml was added to the nematodes during subculturing to avoid contaminating the fresh cultures.

Panagrellus silusiae is an ovoviviparous animal with five larval stages. The first larval stage (L_1) is intrauterine, but the remaining stages (L_2, L_3, L_4 and adult) are free-living. The different stages were identified by using the average lengths of worms, based on the method of Gysels and van der Haegen (1962).

After sample collection, the nematodes were separated by age using the glass

microbead technique outlined by Samoiloff and Pasternak (1969). Since this procedure only separated the youngest free-swimming larval stage, it was necessary to obtain as many L_2 worms as possible. Therefore, seven-day-old cultures were placed in the dark for 16 hr prior to sample collection, to induce the nematodes to reproduce, since *P. silusisc* tends to copulate more often while in darkness, thus producing more L_2 larvae.

When later stages were studied, the L_2 larvae were allowed to molt at 23–25° C to a more advanced stage of the life cycle. Following the work of Chow and Pasternak (1969), the L_2 larvae were added to petri dishes containing 10 ml of clear 1% barley solution, so that the ensuing growth to maturation would be highly synchronous. In the barley solution, L_3 larvae were obtained in 24 hr, L_4 in 48 hr and adults in 72 hr.

The nematodes were also kept in the 1% barley solution until they were 10, 15, 20, and 25 days old, in order to study the aging adult worms. This part of the study was conducted at 5° C, which allowed the adults to age at a somewhat slower rate than normal, but not to reproduce. Therefore, new L_2 larvae could not be born into the age-synchronized cultures. Every 24 hr, one ml of fresh barley solution was added to the petri dishes in each experiment, to offer fresh nutrients.

The nematodes to be studied were concentrated by centrifugation and ground with a Foredom tissue grinder in an ice-cold container to minimize the denaturing of the isozymes. In each experiment, the protein content of the samples was determined using the method of Lowry, Rosebrough, Farr, and Randall (1951). Polyacrylamide gel electrophoresis, based on the methods of Davis (1964) and Ornstein (1964) was used in this study. The following technique changes were made. The bridge buffer used was an 0.01 M histidine-NaOH buffer of pH 7.5, which was suggested by Robinson (1972). The gels were 10% acrylamide and were run at 6° C at 4 mAmp/tube. The sites of acid phosphatase on the gels were determined using the reaction method of Barka (1961). Electrophoretic mobility (E_f) values were determined directly from the gels.

Densitometric tracings of the gels were made immediately after the end of incubation, at 515 nm. The relative activity of each peak was calculated by dividing the peak height by the μg of protein applied to the gel (Bolla, Weinstein and Lou, 1974).

Triton X-100, a detergent which disrupts lysosomal membranes, was utilized to determine the amount of membrane bound and unbound acid phosphatase in the different nematode stages. Modifying the procedure of Meany, Gahan and Maggi (1967), Triton X-100 was added to a mixture of the stages of *P. silusiae* using six different methods of introduction and the following concentrations: 0.1%, 0.5%, 1.0%, 2.5%, 5.0%, 10%, 25%, 50%, and 100%. The six procedures for adding the detergent are as follows: immediately before the nematodes were ground for electrophoresis; 10 min before grinding the nematodes; 10 min at 37° C before grinding the tissue for electrophoretic experimentation; immediately after grinding the nematodes; to pre-ground tissue 10 min before experimentation; and to pre-ground nematodes 10 min at 37° C before running electrophoresis. Each concentration of Triton X-100 was added to the worms in all six of the procedures. Each result compared with the appropriate control

of distilled water added to the homogenate, and equal portions of nematodes and detergent were used in each case.

To determine the quantity of acid phosphatase liberated in each trial, the Sigma total acid phosphatase test (Sigma Technical Bulletin No. 104, 1963, Sigma Chemical Company, St. Louis, Missouri) was run on a sample from each test, at 410 nm on a spectrophotometer. The percentage of transmittance was converted to Sigma units/ml of acid phosphatase, using a standard curve based on para-nitrophenol.

To determine which structures within *P. silusiae* contain acid phosphatase, a light microscopic study was done on each larval stage. The tissue was fixed in 1 : 10 commercial formalin for 1 hr, and dehydrated in an ascending series of ethanols following the procedure of Jensen (1962). The worms were infiltrated with paraffin, positioned in paraffin blocks and sectioned at 10 μ. The sectioned tissue was affixed to glass slides and stained using the acid phosphatase-lead sulfate procedure of Gomori (1952), which was modified by Jensen (1956). The control for this study was heat-killed tissue (*i.e.,* boiled in distilled water for 5 min) carried through the entire staining process.

Results

This study showed that there is a relationship between changes in acid phosphatase activity and life cycle stages in *Panagrellus silusiae.* After measuring the electrophoretic mobility (E_f) values of the stained bands on each gel, it was determined that ten separate and distinct isoenzymes actually existed. The L_2 and L_3 stages each showed four isozyme bands on each gel. Five separate isozymes were present in the L_4, and six distinct bands were found on the gels of the 6-day, 10-day, 15-day, and 20-day-old nematodes. The 25-day-old nematodes showed seven isozymes of acid phosphatase, which was the largest number present in any stage of the life cycle. Only two of the ten isoenzymes were present in all eight of the stages studied. The average E_f values are listed in Table I. Isozyme 10 always travelled beyond the tracking dye, therefore resulting in an E_f value of greater than 1.0.

Table I

Average electrophoretic mobility (E_f) values. A dashed line indicates an absence of the isozyme at that stage.

Stage	Band numbers									
	1	2	3	4	5	6	7	8	9	10
L_2	—	0.1176	—	0.2581	—	0.3521	—	—	—	1.1427
L_3	—	0.1077	—	—	—	0.3529	—	—	0.6452	1.1385
L_4	—	0.1176	—	—	—	0.3516	0.5156	—	0.6464	1.1406
6 days	—	0.1270	—	—	0.3143	0.3498	0.5178	—	0.6395	1.1471
10 days	0.0366	—	—	—	0.3171	0.3540	0.5143	—	0.6402	1.1427
15 days	0.0342	—	0.1370	—	0.3093	0.3501	—	—	0.6461	1.1398
20 days	0.0328	—	0.1313	—	0.3099	0.3521	—	—	0.6380	1.1406
25 days	0.0351	—	0.1405	—	0.3170	0.3513	—	0.5956	0.6453	1.1385

The isozymes of acid phosphatase stained in one of three ways on the gels. The bands appeared either red, faint red, or yellow in color. By conducting a Sigma total acid phosphatase test on each individual band, it was determined that each colored band was truly acid phosphatase. A piece of blank gel was used as a standard.

Densitometric tracings were made of the electrophoretic gels from each age group (Fig. 1 and 2). The relative activities of each separated isozymes were

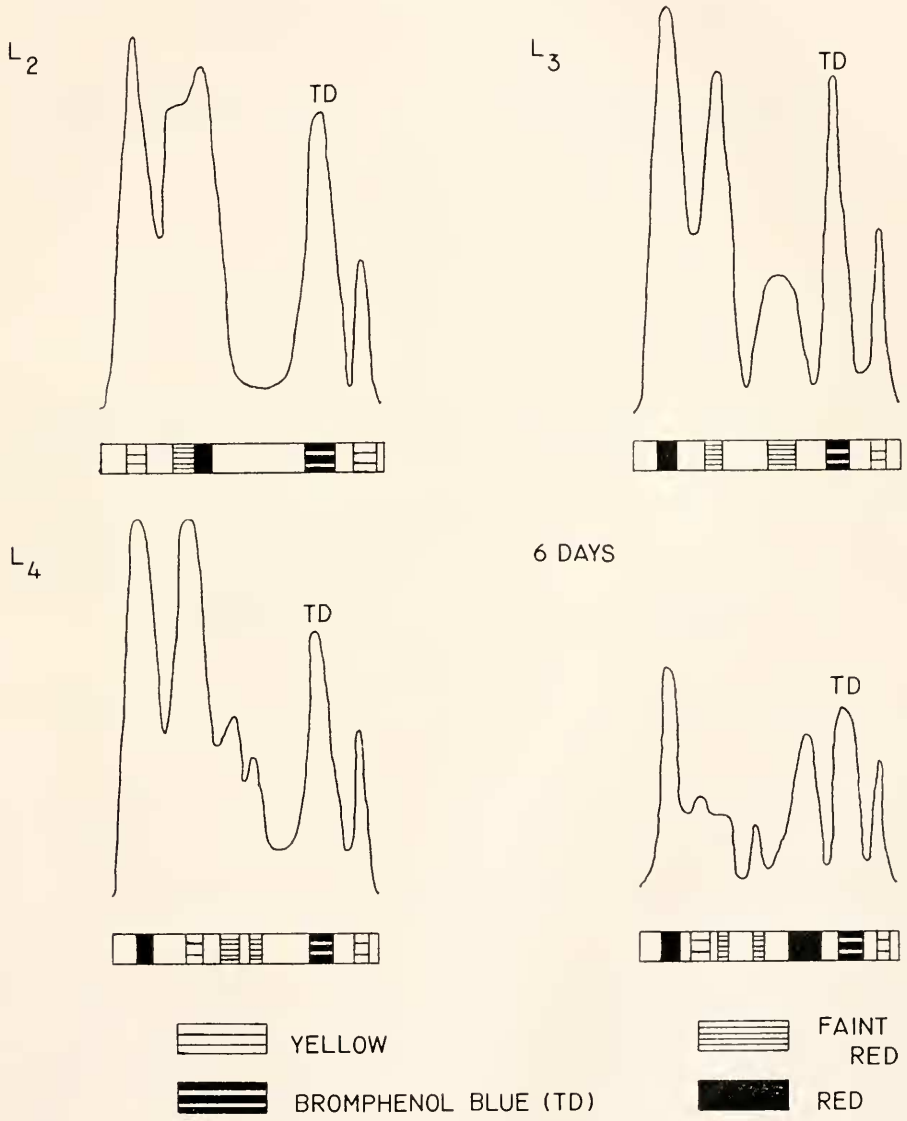

FIGURE 1. Densitometric record of acid phosphatase in L_2, L_3, L_4, and 6-day-old *Panagrellus silusiae*.

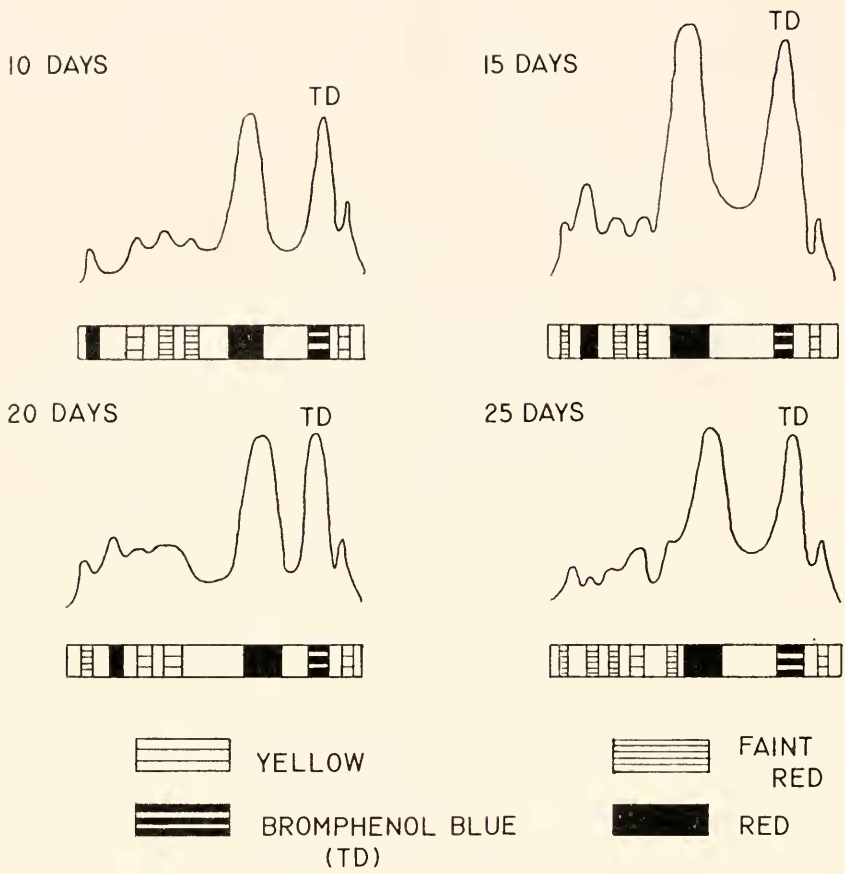

FIGURE 2. Densitometric record of acid phosphatase in 10-day, 15-day, 20-day, and 25-day-old *Panagrellus silusiae*.

calculated directly from the tracings. The values obtained appear in Table II. By studying these values and the densitometric tracings, it can be seen that the individual isozymes increase or decrease in relative activity in correlation with the life cycle stage of the nematode. However, as the nematode cycle progresses, there is a general decrease in the relative activity of the enzyme itself, while there is a concurrent increase in the number of isozymes present.

The results of the Triton X-100 study are listed in Table III. Method I (added and ground immediately) liberated no more than 2% acid phosphatase than the distilled water control, while method II (added and ground after 10 min) showed no increase in the level of acid phosphatase obtained. Method III (added and ground after 10 min at 37° C) and method IV (added immediately to ground tissue) yielded no more than a 3% increase in acid phosphatase activity. Method V (ground 10 min after it was added) showed no increase in the enzyme level, and method VI (ground 10 min after it was added at 37° C)

TABLE II

Relative activity of acid phosphatase (peak height/μg protein applied to gel). A dashed line indicates an absence of the isozyme at that stage.

Stage	Peak numbers									
	1	2	3	4	5	6	7	8	9	10
L_2	—	2.50	—	2.02	—	2.26	—	—	—	1.07
L_3	—	2.73	—	—	—	2.32	—	—	0.95	1.28
L_4	—	2.62	—	—	—	2.62	1.25	—	1.04	1.18
6 days	—	1.57	—	—	0.67	0.50	0.47	—	1.11	0.95
10 days	0.22	—	—	—	0.35	0.37	0.33	—	1.11	0.50
15 days	0.30	—	0.56	—	0.35	0.35	—	—	1.54	0.37
20 days	0.24	—	0.38	—	0.30	0.34	—	—	1.04	0.35
25 days	0.22	—	0.16	—	0.18	0.30	—	0.32	1.03	0.26

liberated no more than 1% acid phosphatase than the control. The results of these six trials indicate that only a negligible amount of acid phosphatase is bound to membranes within the cells.

In order to determine what structures within *Panagrellus silusiae* contain acid phosphatase, the nematodes were specifically stained for the enzyme, and studied using light microscopy. The controls of heat-killed tissue were run for each stage to prove that any staining was truly due to the presence of acid phosphatase and not to something in the staining process itself. In the experimental studies, any structure that appeared black in color contained acid phosphatase.

Only the digestive tract stained lightly in the L_2 stage. The L_3 stage stained lightly throughout the length of its body and the faint line of the intestine was again visible. This indicates that these two stages contain small amounts

TABLE III

Effect of concentration of Triton X-100 on acid phosphatase activities using p-nitrophenol as a substrate (values expressed in Sigma units/ml of acid phosphatase).

Conc. of Triton X-100	Method used					
	I Added and ground immediately	II Added and ground after 10 min	III Added and ground after 10 min (37°C)	IV Added immediately to ground tissue	V Ground 10 min after it was added (37°C)	VI Ground 10 min after it was added (37°C)
0.0%	0.40	0.34	0.39	0.54	0.45	0.51
0.1%	0.41	0.34	0.34	0.55	0.44	0.45
0.5%	0.42	0.32	0.38	0.57	0.39	0.44
1.0%	0.40	0.30	0.39	0.57	0.39	0.50
2.5%	0.35	0.27	0.41	0.52	0.40	0.50
5.0%	0.41	0.31	0.42	0.58	0.43	0.52
10%	0.35	0.29	0.40	0.49	0.44	0.48
25%	0.39	0.27	0.39	0.52	0.44	0.44
50%	0.40	0.31	0.34	0.50	0.40	0.47
100%	0.35	0.30	0.35	0.55	0.44	0.51

of the enzyme. In the L_4 stage, a large amount of staining occurred. For the first time, the esophagus and intestine stained darkly, indicating that large amounts of acid phosphatase were present. Also, the immature gonads, which begin to develop in this stage, stained positively for the enzyme. A number of structures stained in the adult stage, including the entire gastrointestinal tract, the fully developed reproductive system, the excretory canals and eggs within the bodies of sexually mature adult females. L_1 worms waiting to emerge from the bodies of adult females also stained lightly, indicating the presence of a small amount of acid phosphatase.

Discussion

For the data to be meaningful, both the unbound and membrane-bound isozymes of acid phosphatase have to be considered. Since the unbound isozymes could be assayed, Triton X-100 was selected to release those isozymes bound to membranes within the cells. Depending on the method of introduction and concentration of the detergent used, no more than 3% of the total acid phosphatase present in *P. silusiae* was found to be bound by membranes, meaning 97% of the enzyme could be assayed without the use of Triton X-100. Therefore, the amount of bound acid phosphatase was considered negligible, and the use of Triton X-100 was abandoned.

The results of the electrophoretic and densitometric studies indicate that there is a relationship between acid phosphatase levels and specific life cycle stages in *Panagrellus silusiae*. The changes exhibited by acid phosphatase probably result from the involvement of several molecular subunits (isozymes) in the activity of the enzyme.

By studying Table II, it can be seen that isozymes 1, 3, and 5 were only present in the adult stages, indicating they may be connected with the onset of maturity. Isozyme 2 appeared only in the larval stages, indicating involvement with the development of the nematode, instead of the later stages. Because isozyme 4 was only present in the L_2 stage, it seems to be related to some early development in the young larvae. Isozyme 6 was present throughout the entire life cycle. Its relative activity peaked in the L_4 stage, dropped by almost 80% in the molt to the adult stage, and continued to drop during the rest of the life cycle, indicating a greater involvement with early development than with maturation. Perhaps it is involved with the onset of gonadogenesis, since its activity peaks during the stage when this process begins. The seventh isozyme was present briefly in the middle of the life cycle, indicating that it is involved with the onset of development of some particular structures and disappears upon their completion. The eighth isozyme may be involved with the final aspects of aging since it appeared only in the 25-day-old nematodes. Isozyme 9 may be connected with the aging process, since its relative activity peaked in the 15-day-old worms. The tenth isozyme travelled beyond the tracking dye in every stage of the life cycle. This indicates that this isozyme might be of a molecular weight less than that of the bromphenol blue tracking dye, and may be involved in both the early and late stages, since it is always present. However, charge and other factors involved in electrophoresis might also have caused such an occurrence.

The number of isozymes of acid phosphatase increases during the life span in *P. silusiae,* but the relative activities of the different isozymes peak at different stages, while the overall enzyme activity decreases with maturation. These results are consistent with the findings of Erlanger and Gershon (1970) on *T. aceti* and Bolla *et al.* (1974) on *N. brasiliensis,* who concluded that these biochemical changes correlate with the morphological and physical changes that occur during the stages of development and aging throughout the nematode's life cycle.

The isozyme bands on the electrophoretic gels appeared either red, faint red, or yellow in color. The difference between the two shades of red is explained by the fact that those isozymes that stained faint red were always of a lesser activity than those that stained red on the same gel, indicating that there was less of each faint staining isozyme than of those which stained darker. The yellow bands are explained by MacIntyre (1971) who studied the staining reactions used in this research. He found that acid phosphatase ordinarily combines with two molecules of fast garnet, forming a red dicoupled colored complex. However, this usually spontaneous reaction sometimes does not occur to completion, which leaves the acid phosphatase in a yellow, monocoupled colored complex, with one molecule of fast garnet.

From the light microscopic study, it can be seen that the L_2 and L_3 stages show very little staining for acid phosphatase, indicating a low activity of the enzymes. However, the electrophoretic and densitometric studies indicated high levels of activity but few isozymes at these stages. Perhaps some isozymes may not be stained by the histochemical technique used. Due to these somewhat conflicting results, further study of these two larval stages is necessary. Complete staining of the gastrointestinal tract and developing gonads was obvious in the L_4 stage. In the adult stage, the digestive tract, excretory canals, reproductive system, and eggs and unborn L_1 worms in the bodies of mature females, all stained positively for acid phosphatase. This microscopic study therefore indicates that acid phosphatase is present in high concentrations in the digestive, excretory, and reproductive systems of *Panagrellus silusiae,* which is consistent with previous findings in other lower metazoans (Cesari, 1974).

Cristofalo, Parris and Kritchevsky (1967) hypothesized that with increased age, acid phosphatase activity gradually shifts the equilibrium in the cell away from the synthesis and towards catabolism, thus resulting in a general deterioration of the cells. The data indicates that there is a specific change in the isozymes of acid phosphatase which corresponds to the stages in the life cycle. Acid phosphatase isozymes appear to vary with the age of the nematodes, as discussed in the Gershon (1970) model.

Summary

This study showed that there is a relationship between acid phosphatase levels and life cycle stages in the nematode, *Panagrellus silusiae.* Ten different isozymes of acid phosphatase were separated electrophoretically. Relative activity peaked at different stages in the life cycle for the different isozymes. Later in the life cycle, there is a general decrease in the relative activity of acid phosphatase itself, while there is a concurrent increase in the number of isozymes present. At least

97% of the acid phosphatase in *P. silusiae* is soluble (unbound). Acid phosphatase appears to be present in large quantities in the entire gastrointestinal tract, the excretory canals, and the reproductive system of mature *Panagrellus silusiae*.

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